Exhibit 4

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Immune modulation with high-dose heat-shock protein gp96: therapy of murine autoimmune diabetes and encephalomyelitis

Rajiv Y. Chandawarkar, Mihir S. Wagh, Joseph T. Kovalchin and **Pramod Srivastava**

Center for Immunotherapy of Cancer and Infectious Diseases, University of Connecticut School of Medicine, Farmington, CT 06030-1601, USA

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Abstract

Immunization with heat-shock protein (HSP) gp96 elicits protective immunity to the cancer or virus-infected cells from which it is derived. Low doses of gp96 generate immunity, while doses 10 times the immunizing dose do not. We show here that injection of high doses of gp96 generates CD4+ T cells that down-regulate a variety of ongoing immune responses. Immunization with high doses of gp96 prevents myelin basic protein- or proteolipid protein-induced autoimmune encephalomyelitis in SJL mice and the onset of diabetes in non-obese diabetic mice. The suppression of immune response can be adoptively transferred with CD4+ cells and does not partition with the CD25 phenotype. The immunomodulatory properties of gp96 (and possibly other HSP) may be used for antigen-specific activation or suppression of cellular immune responses. The latter may form the basis for novel immunotherapies for autoimmune diseases.

Introduction

Immunization of mice with heat-shock protein (HSP)–peptide complexes has been shown to elicit CD8+ and CD4+ T cell responses specific to the HSP-chaperoned antigenic peptides, and to the cells that present those peptides in the context of their MHC molecules (1). The quantity of HSP-chaperoned peptide required to generate immunity is extremely small; femtomoles are generally sufficient (2,3). As HSP–peptide complexes derived from antigenic tissues contain a minute quantity of antigenic peptides in an abundance of self-peptides and as HSP are powerful adjuvants (2), the dangers of eliciting autoimmunity require consideration. For this purpose, we monitored mice immunized with gp96–peptide complexes derived from normal tissues for signs of pathological autoimmunity. No such signs were detected (4). Normal mouse blood contains low titers of IgD and IgM antibodies to hsp70 and gp96, but these titers do not increase with immunization (4). Several hundred cancer patients immunized with gp96–peptide complexes have been monitored without signs of pathological autoimmunity (5,6).

Paradoxically, immunization with large doses of gp96 has been shown to suppress the immune response rather than provoking autoimmunity (7,8). Less than 5 μg gp96–peptide complexes is insufficient to immunize, ~10 μg is sufficient to cause tumor rejection and ~100 μg fails to elicit tumor immunity. The biological basis of this dose window of immunogenicity is not yet understood. In this paper, we have investigated this phenomenon using murine cancer models as well as murine models of human multiple sclerosis and Type 1 diabetes. We report that, depending upon the dose of immunization used, gp96 may elicit an antigen-specific immune response (as shown previously) or down-regulate pre-existing pathological autoimmune responses through the generation of immunoregulatory CD4+ T cells.

Methods

Mice and cell lines

Female BALB/cJ and NOD/LtJ mice (6–8 weeks old) were obtained from the Jackson Laboratory (Bar Harbor, ME) and were housed in the Animal Facility at Fordham University, Department of Biological Sciences or the University of Connecticut School of Medicine. Female SJL mice (10–12 weeks of age) obtained from the Jackson Laboratory were used for the studies on experimental autoimmune encephalomyelitis (EAE). The Meth A tumor cell line was obtained from

Correspondence to: P. Srivastava; E-mail: Srivastava@nso2.uchc.edu

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our tumor bank and grown as ascites in BALB/cJ mice. All work with mice was approved by the IACUCs of the respective institutions.

Purification of gp96

gp96 was purified as described (8). The preparations were homogeneous as judged by SDS–PAGE and were tested for endotoxin content by the limulus amebocyte lysate assay (Biowhittaker, Walkersville, MD) following the manufacturers' protocol.

Immunization, tumor challenge and tumor measurements

These procedures were carried out as described (8).

Induction and monitoring of EAE

EAE was induced by injection of bovine myelin basic protein (MBP; Sigma, St Louis, MO) dissolved in PBS (8 mg/ml) and emulsified with an equal volume of complete Freund's adjuvant (CFA; Sigma). Each mouse received 0.1 ml of the emulsion (containing 400 μg of bovine MBP) divided in two sites on the shaved flank and administered s.c. Induction of EAE with proteolipid protein (PLP; sequence HSLGKWLGHP-DGF, a gift from Dr V. Kuchroo of Harvard University) was carried out by immunizing mice with PLP dissolved in PBS and emulsified with an equal volume of CFA. Each mouse received 50 μg of PLP divided in two sites on the shaved flank and administered s.c. Pertussis toxin (100 μl) was administered into the retro-orbital venous plexus 2 days following the administration of PLP. Animals were observed for clinical signs of disease. Clinical severity was graded as follows (score 0–6): 0 = no paralysis, 1 = tail paralysis, 2 = impaired gait, 3 = paralysis of both hind limbs, 4 = paralysis of all four limbs, 5 = moribund and 6 = death.

Monitoring of diabetes

Two separate urine glucose estimations were made prior to immunization and repeated weekly thereafter, using Diastix (Fisher, Pittsburgh, PA). Mice were considered clinically diabetic if urine glucose levels reached 1000 mg/dl or higher.

Histology

The pancreas of non-obese diabetic (NOD) mice were dissected and fixed in 10% buffered formalin. Histological analysis (staining with hematoxylin & eosin and anti-insulin antibody) was performed by An-Path Services (Farmington, CT).

Adoptive transfer of splenocytes

Spleens were harvested from donor mice and mechanically pulverized. Red blood cells were removed by incubation of the total cells with a filtered solution containing ammonium chloride. The residual cells were labeled with MACS micro-beads conjugated to rat anti-mouse $CD4^+$ or $CD8^+$ antibodies per the manufacturer's protocol and loaded onto MS columns or the AutoMACS separation unit (Miltenyi Biotec, Auburn, CA) for positive selection of $CD4^+$ or $CD8^+$ T cells.

For further separation of $CD4^+CD25^+$ and $CD4^+CD25^-$ the following steps were added. Cell pellets were resuspended in Fc block and incubated for 5 min at 4°C. Cells were then incubated with phycoerythrin (PE)-labeled anti-$CD4^+$ GK1.5 antibody and FITC-labeled anti-CD25 7D4 antibody (PharMingen, San Diego, CA) for 30 min at 4°C at concentrations following the manufacturer's protocols. Cells were then sorted on the FACStar advantage (Becton Dickenson, Mountain View, CA) for double-positive and PE single-positive populations. Sample purity was checked by flow cytometry after cell sorting and fractionation. Cell populations were counted and stained with Trypan blue to assess cell viability. Cell numbers for each individual experiment were as described in the results. Cells were suspended in 200 μl of PBS and adoptively transferred by i.v. injection via the retro-orbital venous plexus. Anesthetics (ketamine 2.0 mg and xylazine 0.1 mg) were used i.p. to minimize animal discomfort.

Statistical analysis

Statistical significance was determined using a non-parametric Mann–Whitney test. *P* values were calculated using Minitab statistical software.

Results

Immunization with high-dose gp96 suppresses tumor immunity

BALB/cJ mice (6–8 weeks of age) were immunized s.c. with 10 μg gp96 (two doses 1 week apart) obtained from Meth A fibrosarcoma or normal liver. One week following the last immunization, the mice were challenged with 100,000 live Meth A cells injected intradermally. Tumor growth was recorded periodically. Consistent with several previous studies (7,8), it was noted (Fig. 1) that Meth A gp96 was effective in eliciting tumor regression, whereas liver gp96 was not. In contrast, high-dose (100 μg) Meth A gp96 was unable to immunize and elicit protective immunity, confirming our previous results (7). In that previous publication we demonstrated that the inability of high-dose gp96 to immunize arose from its immunosuppressive properties and that the suppression could be adoptively transferred by $CD4^+$ T cells from mice immunized with high-dose Meth A-derived gp96. We next sought to determine whether the source of gp96 was important in suppressing the generation of an anti-tumor immune response. High-dose (100 μg) gp96, consisting of an immunizing dose of 10 μg Meth A gp96 plus an additional 90 μg of liver-derived gp96, was used to immunize mice prior to challenge with live Meth A cells. The results show that mice immunized with an immunizing (10 μg) dose of Meth A gp96 mixed with a high dose (90 μg) of liver- or Meth A-derived gp96 failed to be immunized (Fig. 1). While only Meth A gp96 was effective in generating protective immunity against Meth A, both Meth A- and liver-derived gp96 were equally effective in suppressing immunity when given at high doses.

The ability of Meth A-derived gp96 as well as liver-derived gp96 in high doses to suppress tumor immunity as shown here may appear to conflict with the data shown by us previously (7). We showed previously in fig. 3 of (7) that antigen-specific suppression elicited by high-dose gp96 could only be elicited by gp96 derived from Meth A, but not liver. The discrepancy results from differences in the experimental design and is illuminating. In that study, resection of a growing tumor was the immunizing stimulus and we tested if previous administration

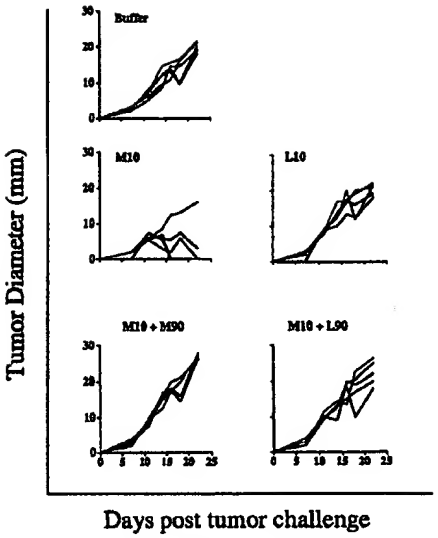

Fig. 1. Lack of source specificity of gp96 for mediating down-regulation of tumor immunity. BALB/cJ mice (6–8 weeks of age) were immunized s.c. with PBS, optimal immunizing dose (10 μg) of Meth A gp96 (M10) or high-dose (100 μg) Meth A gp96 (M10 + M90). Liver-derived gp96 was tested as a control in the immunizing dose of 10 μg (L10), or in high dose, mixed with an immunizing dose of Meth A gp96 (10 μg Meth A gp96 + 90 μg liver-derived gp96 = 100 μg total gp96, M10 + L90). Note that while only Meth A-derived gp96 was effective in eliciting tumor immunity, high-dose Meth A or liver gp96 suppressed immunity.

(previous to resection) of high-dose Meth A gp96 or high-dose liver gp96 could abrogate the resection-elicited immunity. High-dose Meth A gp96 was found to be suppressive, but high-dose liver gp96 was not, leading us to conclude that cognate gp96 at high dose was required for suppression. In light of our present data (Fig. 1, this study), it is clear that that the interpretation in 1999 was incorrect. High-dose Meth A gp96 was suppressive because it contained the immunizing dose of Meth A gp96 and the suppressive larger dose of Meth A gp96. The high-dose liver gp96 did not suppress, not because it is not immunosuppressive, but because it did not contain the low immunizing dose of Meth A gp96. As we demonstrate in the following section, as long as the specific immunizing dose is given before the suppressive high-dose gp96, the high-dose gp96 could be from any source.

The immunosuppressive activity of high-dose gp96 requires prior existence of an immune response

The temporal relationship between immunization and suppression was explored [Fig. 2a (experimental plan) and b (data)]. Mice were immunized with a tumor-protective dose of Meth A gp96, twice 1 week apart and evenly divided into groups. Some groups were administered high-dose gp96 before immunization, while others received it at various periods after immunization. Thus, the timing of administration of high-dose gp96 was varied with respect to the timing of the

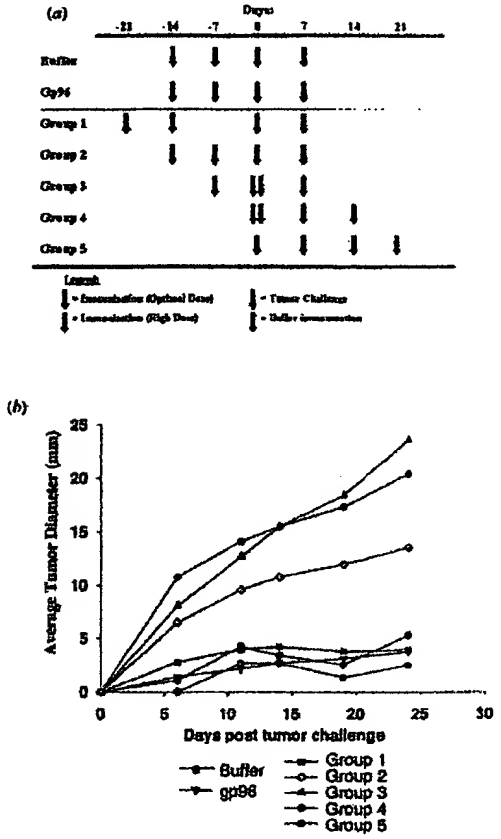

Fig. 2. High-dose gp96-mediated immunological suppression requires a pre-existing immune response. (a) Study design. Animals were immunized with a protective dose of gp96 (purple arrow) and/or a high suppressive dose of gp96 (red arrow) at various times as shown. All animals were challenged with 100,000 live Meth A tumor cells intradermally (green arrow at the time indicated). Animals immunized with buffer (blue arrow) served as controls. (b) Tumor rejection data from (a). Each line represents the kinetics of tumor growth in the group indicated; the average tumor diameter of five or more mice per group at each time point was used to generate the kinetics.

protective dose. All mice were challenged with live Meth A cells and tumor growth was monitored.

Results (Fig. 2b) show that mice immunized with an optimal dose of gp96 remained tumor-immune, while buffer-immunized mice succumbed to the challenge. Suppression of immunity became evident only in mice that received the high-dose gp96 *after* receiving the tumor-protective dose of gp96. Significant differences in the extent of suppression were observed among such mice: mice receiving a tumor-protective dose 21 days prior to the suppressive dose could not be suppressed and remained tumor immune (Group 1). Suppression became evident as the interval between the timing of protective and suppressive doses became shorter

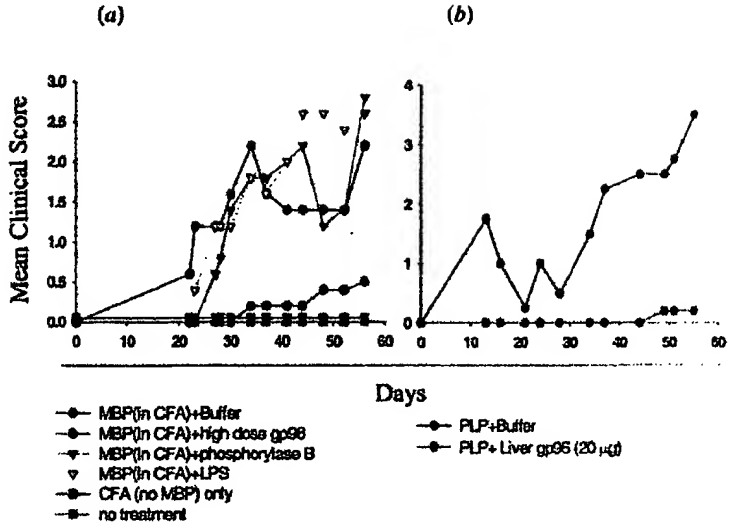

Fig. 3. Protection of SJL mice against EAE with high-dose gp96. EAE was induced in SJL mice by injecting MBP (a) or PLP (b) in CFA. The mice were injected with MBP or PLP as described in Methods and treated with high-dose gp96 or buffer the next day, and once again 1 week later. Animals treated with phosphorylase b or LPS served as controls. Disease scores represent average scores of five mice per group on the days indicated. *P* values for (a): $P < 0.0001$ for comparison with buffer; $P < 0.0046$ for comparison with phosphorylase b, $P < 0.0012$ for comparison with LPS; for (b): $P < 0.0001$.

(Groups 2 and 3). Maximal suppression was seen when the protective and suppressive doses were given at the same time (Group 3). Mice that received high-dose gp96 before being immunized with (Group 5) immunogenic doses of gp96 remained tumor-immune, i.e. were not suppressed. A comparison of Groups 3 and 4 is illustrative in this regard. Mice in both groups received two immunizations with the protective dose of gp96 and were challenged with live tumor cells 1 week after the second immunization. The only difference between the groups is that mice in Group 3 received the high-dose gp96 with the second protective dose (i.e. after they had been primed already by the first protective dose), while mice in Group 4 received high-dose gp96 with the very first protective dose (i.e. when they were not yet primed). The single high-dose gp96 immunization in Group 3 was given after the mice had received the first immunizing dose. Thus, this high-dose immunization was able to act on the immune response generated and was able to suppress it. However, in Group 4, while one immunizing dose was administered with the high-dose gp96, the second dose was given after the high dose. Thus, we speculate that while the T cells activated as a result of the first immunization were suppressed by high-dose gp96 as in Group 3, the T cells newly activated by the second immunization were not and went on to mediate tumor immunity, as in Group 4. Thus, the difference in timing of administration of the high-dose gp96 with respect to the immunizing stimulus has binary consequences for protective immunity.

High-dose gp96 inhibits onset of EAE

Immunization of SJL mice with MBP induces EAE (9) and similarities of this disease to human multiple sclerosis have been noted. Since EAE pathology is T cell mediated, the ability of high-dose gp96 to down-regulate autoimmune pathology in EAE was tested.

SJL mice were immunized with MBP as described in Methods and were treated with high-dose gp96 derived from normal mouse livers. As a control, MBP-immunized mice were treated with the unrelated protein phosphorylase b. Mice were scored for paralysis as described in Methods. It was observed (Fig. 3a) that 60–75% of mice immunized with MBP that were untreated, or treated with saline, lipopolysaccharide (LPS) or phosphorylase b developed progressive paralysis of all four limbs and had to be euthanized by day 60 post-MBP immunization. In contrast, only 20–40% mice treated with high-dose gp96 were paralyzed ($P < 0.0001$ for comparison with buffer; $P < 0.0046$ for comparison with phosphorylase b; $P < 0.0012$ for comparison with LPS).

In addition to MBP, immunization with PLP has been shown to induce EAE (10). The effect of treatment with high-dose gp96 was tested in this model. The results (Fig. 3b) were nearly identical to those seen with high-dose gp96 treatment in the MBP model, in that all PLP-immunized mice succumbed to the disease, while the majority of PLP-immunized mice treated with high-dose gp96 were protected from it ($P < 0.0001$).

Immunization with high-doses of gp96 inhibits diabetes in NOD mice

The generality of the idea that high-dose gp96 can mediate immunological suppression was tested further by extending the studies to NOD mice (11). Such mice develop insulitis spontaneously, starting at ~5 weeks of age and clinical diabetes starting at ~10–12 weeks of age. By 24 weeks of age,

>80% female mice develop diabetes. Pathogenesis is due to autoreactive CD4+ and CD8+ T cells against islet cell antigens.

NOD mice (6–8 weeks of age) were treated with gp96 obtained from liver or pancreas of NOD mice, at one of three doses (10, 25 and 100 μg per s.c. injection, two treatments, 1 week apart). The gp96 preparations used in this study were free of detectable levels of LPS (0.1 EU/ml). Control animals were either treated with PBS or were left untreated, as indicated. All mice were monitored for onset of diabetes by measuring urine sugar as described in Methods.

No difference in the kinetics of onset of disease was detected among untreated mice and mice treated with PBS, 10 or 25 μg pancreas- or liver-derived gp96 (Fig. 4a). Mice in these groups began to develop diabetes between 10 and 14 weeks of age, and all mice were diabetic by 20 weeks of age. In contrast to the mice immunized with 10 or 25 μg gp96, 60–80% of the mice immunized with 100 μg gp96 derived from pancreas or liver respectively remained free from disease for the entire period observed (6 months of age) (Fig. 4b, $P < 0.0001$ for comparison of buffer with liver gp96; $P < 0.008$ for comparison of buffer with pancreas gp96). There was no significant difference in activity among gp96 derived from pancreas or liver. The suppression of diabetogenesis by high-dose gp96 has been reproducibly observed and the protection has been shown to remain effective for 8 months or longer (Fig. 4b). Control proteins such as ovalbumin, β-galactosidase or phosphorylase b at doses equimolar to 100 μg gp96 did not confer protection (Fig. 4b).

High-dose gp96 protection against onset of diabetes is not due to LPS contamination

NOD mice housed in non-pathogen free colonies show significantly low penetration of autoimmune diabetes (11). Concordantly, ingestion of bacterial LPS reduces the incidence of diabetes in NOD mice. We were concerned about the possibility that trace amounts of LPS may be responsible for the diabetes-suppressive activity of gp96. In order to test this possibility, NOD mice were immunized with a range of quantities of LPS (100 pg to 1 μg LPS or undetectable to >1400 EU) and were monitored for onset of disease (Fig. 4c and d). For comparison, we used gp96 preparations containing undetectable levels of LPS, with a limit of detection of 0.1 EU/ml. Among mice immunized with all three doses of LPS tested, 80% (four of five in each group, altogether 12 of 15) became diabetic by 19 weeks of age. Untreated mice succumbed to disease at an identical incidence rate (8 of 10), while all mice that received high-dose gp96 remained entirely protected from diabetes (0 of 15) (Fig. 4c and d). These observations show that the diabetes-suppressive activity of gp96 does not arise from contaminating LPS; at the same time, they may shed light on the mechanisms through which ingested, but not injected, LPS mediates protection from diabetes in NOD mice.

Histological analysis of pancreas

Histological analysis of the pancreas of healthy untreated or diabetic untreated NOD mice and high-dose gp96-treated NOD mice was undertaken. At 22 weeks of age, the islets of Langerhans of buffer-treated mice were observed to be heavily infiltrated with lymphocytes, while the islets in high-dose gp96-treated mice were infiltrated to a significantly lesser extent and indeed appeared similar to those of 9-week-old naive NOD mice (Fig. 5). Staining of the islets for insulin showed them to be heavily positive for insulin in naive healthy young mice and older mice treated with gp96, but unstained in the case of untreated older mice in whom diabetes had already set in. Interestingly, the gp96-treated mice showed peripheral insulitis in the islets (Fig. 5).

CD4+ T cells are responsible for suppression of diabetes

NOD mice were treated with high-dose gp96 and were observed for 20 weeks without further manipulation. The mice that were protected from diabetes were sacrificed and their spleens fractionated into CD4+ and CD8+ T cells as described in Methods (>95% purity as monitored by FACS analysis, data not shown). As controls, fractionated spleen cells from buffer-treated, pre-diabetic mice (10–12 weeks old) were obtained. The unfractionated, CD4+ (7.5×10^6 cells), CD8+ (4×10^6) or combined CD4+ and CD8+ T cells from gp96-treated or untreated mice were adoptively transferred i.v. into 6- to 8-week-old pre-diabetic NOD mice. Recipient control NOD mice received PBS or were untreated. All recipients were monitored for onset of diabetes. It was observed that 70% of the CD4+ recipients, but none of the CD8+ recipients, were protected from diabetes (Fig. 6a). Mice that received combinations of the two cell types or unfractionated spleen were protected. In contrast, mice receiving any cell type from untreated mice, or PBS or left untreated, developed diabetes with the expected kinetics (Fig. 6b).

The minimum number of CD4+ T cells needed to adoptively transfer protection was determined through titration experiments. As few as 250,000 CD4+ T cells isolated from mice treated with high-dose gp96 were able to transfer protection to 100% of the recipient mice; the same number of CD4+ T cells from buffer-treated mice was unable to accomplish this (data not shown).

Several recent studies have implicated a CD25+ subset of CD4+ T cells in immunosuppressive activity (12,13). CD4+ T cells elicited by treatment of NOD mice with high-dose gp96 were separated into CD25+ and CD25- subsets of ~92 and 97% purity respectively (Fig. 6c), as described in Methods. Both subpopulations, as well as the unfractionated CD4+ T cells, were adoptively transferred into 8- to 9-week-old female NOD mice, which were then monitored for glycosuria. The unfractionated and the fractionated CD25+ and CD25- populations showed identical protective activity against diabetes (80–100% mice protected); corresponding T cell populations from buffer-injected mice did not mediate protection (Fig. 6d).

Discussion

Immunization with high-doses of gp96 is shown here to down-regulate pre-existing active T cell responses in four independent models: tumor immunity, two models of allergic encephalomyelitis and autoimmune diabetes. Immunity generated by gp96 has been shown previously and extensively to be source-specific and dependent on the presence of antigenic peptides (8,14–20). In contrast, the suppression elicited by high-dose gp96 does not require source specificity. gp96 purified from a tumor or normal tissue is equally able to

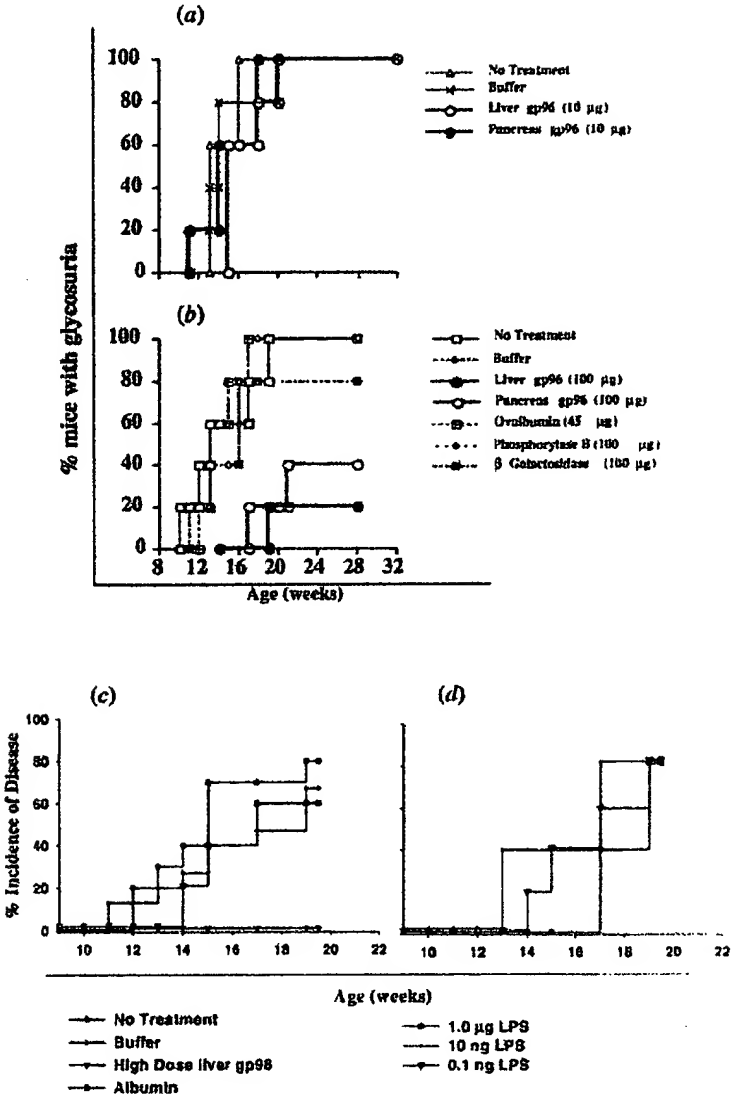

Fig. 4. Protection of NOD mice against diabetes with high-dose gp96. Incidence of glycosuria in NOD mice, 6–8 weeks of age, immunized with pancreas or liver gp96 in varying doses, or with control proteins as indicated (a and b). Animals untreated or treated with buffer served as controls. Mice that were untreated, or treated with 10 µg of pancreas or liver gp96, or buffer developed diabetes (a), while 60–80% of those that received higher doses (100 µg) of gp96 remained free of disease (b). Animals that received 25 µg gp96 showed a kinetics of onset that was indistinguishable from the group that received the 10 µg dose (data not shown). Diabetes developed in almost all animals injected with control proteins (b). ($P < 0.0001$ for comparison of buffer with liver gp96, $P < 0.008$ for comparison of buffer with pancreas gp96.) (c and d) Contaminating LPS in gp96 preparations is not responsible for suppression of diabetes in NOD mice. NOD mice at 9–10 weeks of age were untreated or treated with 100 µg of liver-derived gp96, albumin or buffer (c). Other groups of NOD mice of the same age were treated with titrated doses of LPS as indicated (d).

elicit suppression of tumor immunity or autoimmunity in the case of EAE or diabetes. Thus, the suppressive activity of gp96 can be deduced to reside either in the polypeptide chain itself or in a common peptide associated with gp96 regardless of its source. At present it is not technically feasible to denude gp96 preparations from associated peptides without also

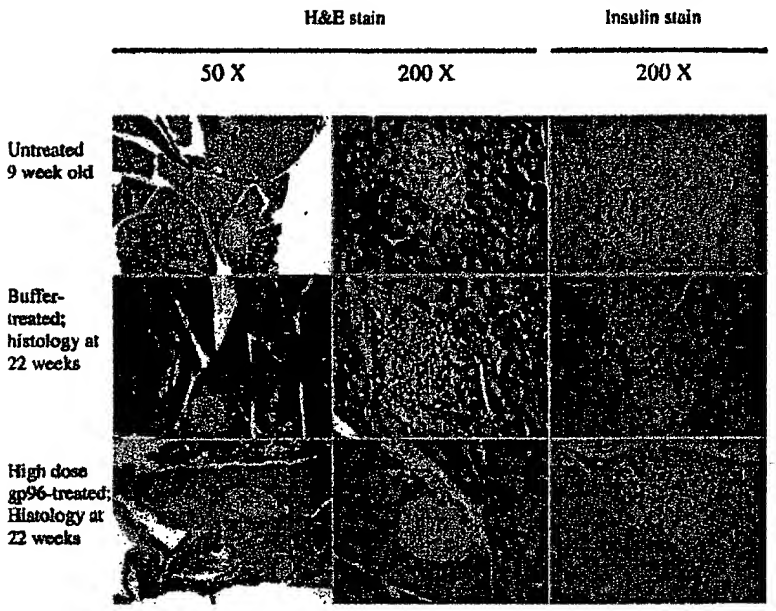

Fig. 5. Histological analysis of the pancreas of untreated NOD mice (9 weeks old), NOD mice treated with buffer at 9 weeks of age and sacrificed at 22 weeks of age, and NOD mice treated with high-dose gp96 at 9 weeks of age and sacrificed at 22 weeks of age. The pancreas was dissected, fixed in formalin and stained with hematoxylin & eosin or insulin. Note the heavy infiltration of lymphocytes in buffer-treated mice (middle panel) at 22 weeks, while no such infiltration is observed at 9 weeks (upper panel). Infiltration is also not seen in 22-week-old mice treated with high-dose gp96 (lower panel). The islets of 9-week-old untreated mice are heavily stained for insulin (upper panel), while insulin production is entirely lost at 22 weeks (middle panel). Mice treated with high-dose gp96, however, show significant insulin production in the islets at 22 weeks of age (lower panel).

denaturing the protein and, thus, it is difficult to distinguish between these possibilities.

The studies aiming to uncover the temporal relationship between immunization and suppression revealed (Fig. 2) that high-dose gp96 was unable to mediate immune suppression in mice where the antigenic stimulus was given following the suppressive dose of gp96. From this, we conclude that suppression of the immune response by high-dose gp96 requires an ongoing immune response as a substrate. These results are also consistent with the observation in the NOD mouse model that treatment of 4- to 5-week-old NOD mice with high-dose gp96 was ineffective at suppressing onset of diabetes, even as 8- to 10-week-old mice treated in the same manner were protected (data not shown). Based on the timing of onset of insulitis (that precedes destruction of β cells), it has been surmized that the autoimmune process in NOD mice does not begin until 6 weeks of age (11). Treatment with suppressive doses of gp96 before that time does not suppress diabetogenesis. While the idea that suppression requires an ongoing immune response that can be suppressed may appear tautological, it indicates clearly that high-dose gp96 does not compromise immune function *per se*, but modulates or causes deviation of pre-existing immunizing circuitry.

Another aspect of the temporal relationship between immunization and suppression requires comment. Suppression is observed to be most effective in recently immunized mice: mice immunized 3 weeks before receiving the high-dose gp96 remained tumor-immune, while mice immunized immediately prior to receiving the high-dose gp96 were immune suppressed. This observation in a single model system, if broadly true, may suggest that the suppression is most effective on activated T cells and that memory T cells may be refractory to it. The cell-surface markers that distinguish between memory and activated T cells (21) may provide a means of discrimination for the suppression mediated by high-dose gp96. At first glance, the observations that mice immunized 3 weeks before receiving the high-dose gp96 remained tumor-immune may appear contradictory to the observation that autoimmunity in NOD mice of 10 weeks of age (in which the autoimmune process began 5–6 weeks earlier) can be suppressed by high-dose gp96; it is clear, however, that the NOD mice contain activated T cells which can be suppressed, while the mice immunized against the tumor 3 weeks earlier only harbor memory T cells.

Suppression of autoimmune response elicited by high-dose gp96 in our present studies is clearly mediated by $CD4^+$ T cells. In a previous publication (7) we demonstrated that the tumor-specific suppression generated by high-dose gp96 could also be adoptively transferred by $CD4^+$ T cells. Regulatory $CD4^+$ T cells have been reported previously in several models of tumor immunity (22) and autoimmunity (23), and our results are consistent with such considerable past evidence. However, a true mechanistic understanding of the

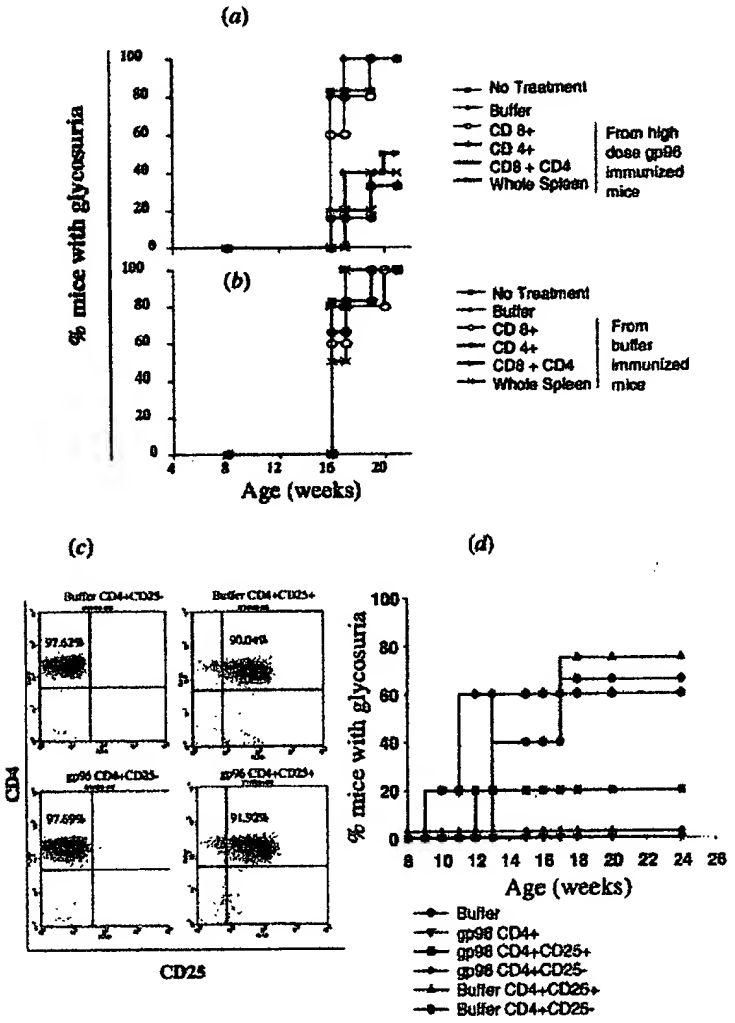

Fig. 6. Cell-surface phenotype of immune-suppressive CD4⁺ T cells. (a and b). Splenocytes (unfractionated, CD4⁺, CD8⁺ or combined CD4⁺ and CD8⁺ T cells) from NOD mice treated with high-dose gp96 and subsequently free of diabetes, or from buffer-treated 10- to 12-week-old non-diabetic mice were adoptively transferred into recipient pre-diabetic NOD mice as shown. Unfractionated, CD4⁺, but not CD8⁺, or combined CD4⁺ and CD8⁺ T cells from high-dose gp96 treated mice were able to protect recipient mice from diabetes (a), while mice that received T cells from untreated mice developed diabetes (b). (c and d) Immunosuppressive activity of CD4⁺ T cells elicited by treatment with high-dose gp96 does not partition with CD25 expression. (c) FACS analysis of fractionated CD4⁺ T cells (CD25⁺ or CD25⁻) from splenocytes obtained from NOD mice, which were treated with high-dose gp96 (and were therefore non-diabetic) or from 10- to 12-week-old buffer-treated mice (which were non-diabetic). (d) Subpopulations of splenic CD4⁺ T cells were adoptively transferred into 8- to 9-week-old female NOD mice which were monitored for glycosuria. Mice receiving 200,000 CD4⁺CD25⁺ were compared to those that received 600,000 CD4⁺25⁻ cells. Selection of these cell numbers was based upon their relative fractions observed during purification of the entire splenic CD4⁺ compartment. However, adoptive transfer of equal numbers of the two subpopulations showed identical results (data not shown).

immune suppression elicited by high-dose gp96 is still lacking and requires experimental pursuit. What is the target cell(s) for high-dose gp96? Interaction of gp96 with antigen-presenting cells (APC) through scavenger receptors CD91 and CD36 (24–26) and Toll-like receptors TLR2 and TLR4 (27) has been documented, and additional receptors have been suggested (28). Studies *in vitro* with effects of increasing concentrations of gp96 on APC function do not reveal a critical threshold at which the APC change qualitatively (S. Basu and P. Srivastava, unpublished). The effects of gp96 on T cells

remain unexplored, although preliminary analyses by von Bonin and co-workers (29,30) show that gp96 molecules can activate T cells directly and regardless of associated peptides, in addition to the peptide-dependent pathway. Such a direct effect of high-dose of gp96 on T cells may provide a window into the mechanisms that mediate the suppression observed in our studies. More recently, Schild and co-workers have reported the presence of gp96 receptors on platelets (31) and other cells (32) of hematopoietic lineage, and it is conceivable that these cells could play a role in high-dose gp96-mediated suppression through elaboration of suppressive cytokines. Whatever the proximal targets, the action eventually converges on generation of suppressor $CD4^+$ T cells that are able to inhibit specific $CD8^+$ T cell responses of a wide variety. Two non-exclusive possibilities may be envisaged. High-dose gp96 may cause 'deviation' of an existing antigen-specific $CD4^+$ response into an antigen-specific suppressor $CD4^+$ population through any of the intermediary steps imagined earlier. The $V\alpha14$–$J\alpha15$ NKT cell-mediated activation of an islet autoantigen-specific T_h2 helper population (elicited by administration of NKT cell ligand α-galactosylceramide) is a useful precedent in this regard (33) as is the demonstration that NKT cells inhibit the function of $CD4^+$ T cells that mediate diabetes in NOD mice (34). Alternatively, immunization with high-dose gp96 may lead to activation of a non-specific super-regulatory $CD4^+$ suppressor population that could inhibit any activated T cell response. The issue rests on whether or not the suppressor $CD4^+$ T cells generated by administration of high-dose gp96 are antigen specific. Although our data do not respond to this aspect formally, the observation that suppression requires a pre-existing immune response prejudices us in the direction of antigen specificity of the suppressor $CD4^+$ T cells generated.

The immunosuppressive activity of $CD4^+$ T cells has been shown to partition into the $CD25^+$ subset in a number of well-studied systems (12,13). This does not appear to be the case with the immunosuppressive activity of $CD4^+$ T cells elicited by immunization with high-dose gp96 (Fig. 6d), as also seen in a number of other systems (35,36). Clearly, there are several roads to suppression and CD25 is the 'road not taken' (37) in this system.

The results described in this study show that high-dose gp96 elicits antigen-specific suppression in a wide array of models of immunity, including autoimmunity. While the broad outlines of the mechanism of suppression are clear, the detailed pathways remain to be elucidated. Understanding these pathways will allow development of reagents that may interact with various elements of this mechanism. Such reagents shall have therapeutic or ameliorative potential for several autoimmune pathologies.

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Abbreviations

APC	antigen-presenting cell
CFA	complete Freund's adjuvant
EAE	experimental allergic encephalomyelitis
HSP	heat-shock protein
LPS	lipopolysaccharide
MBP	myelin basic protein
NOD	non-obese diabetes
PE	phycoerythrin
PLP	proteolipid protein

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